Analysis of the prevalence and diversity of rickettsial species found in Ohio *Amblyomma americanum* ticks, assessed by the analysis of the 17kDa surface antigen gene

A Senior Honors Thesis

Presented in Partial Fulfillment of the Requirements for graduation *with distinction* in Molecular Genetics in the undergraduate colleges of The Ohio State University

by

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June 2006

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INTRODUCTION

Bacteria within the order *Rickettsiales* are obligate, intracellular endosymbionts or parasites that are associated with arthropod hosts as vectors or as reservoirs. Although originally subdivided into three genera, *Coxiella, Rickettsia, and Rochalimaea*, and considered often to be separate from other bacterial groups, the family Rickettsiaceae was recently moved into the α-*Proteobacteria* subgroup. Recent molecular biology techniques such as polymerase chain reaction (PCR) and sequencing, enabled more precise positioning of each genus. Specifically, the 16S rDNA gene was used to place *Rickettsia* with respect to other groups (Weisberg et al, 1989) and to identify the relationships among the various species of *Rickettsia* (Stothard and Fuerst, 1995; Roux and Raoult 1995). Both *Coxiella* and *Rochalimaea* were eventually removed from the family, reducing the family to just *Rickettsia* (Weisburg et al. 1989, Brenner et al. 1993). The genus is classically subdivided into three groups based upon the presence or absence of the outer membrane protein (*OmpA)*: the typhus group (TG), the spotted fever group (SFG), and recently, a small ancestral group comprised of both *R. belli* and *R. canadensis* (Stothard and Fuerst 1995). In addition, the scrub typhus group, represented by a single species, had long been considered to be a part of the genus *Rickettsia*. However, even though it has a very close phylogenetic relationships to *Rickettsia*, the scrub typhus group was removed to its own genus, Orientia, with a single species, *Orientia tsutsugamushi* (Tamura et al. 1995). The relationship among a number of the members of the genus Rickettsia is shown in Figure 1.

Humans serve as accidental hosts for most rickettsial infections, which cause a variety of illnesses worldwide. After being transmitted via an arthropod vector, the

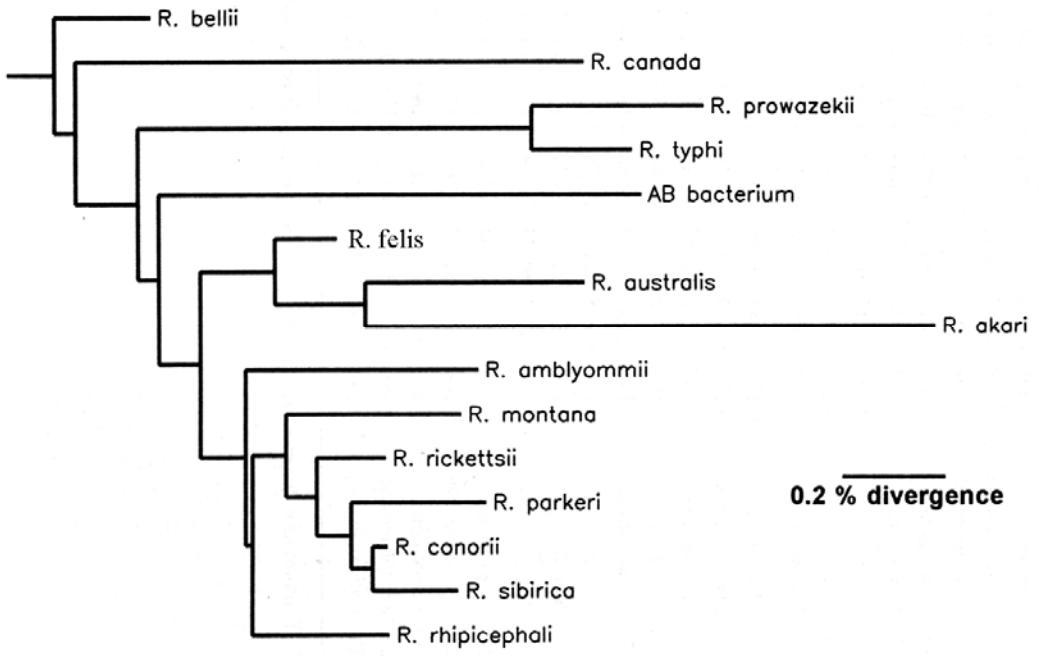

Figure 1. Phylogeny of members of the genus *Rickettsia*. Taken from Stothard and Fuerst (1995)

rickettsial organism's entry into host eukaryotic cells is mediated by both *OmpA* and *OmpB* (Olano, J 2005). A systematic infection then proceeds including such symptoms as fever, headache, and cutaneous eruption (Roux and Raoult 1995).

Human monocytic ehrlichiosis (HME) is a febrile illness that is, at times, fatal. It was first described in the United States in 1986 (Maeda et al. 1987). The etiological agent of HME is the bacteria *Ehrlichia chaffeensis*. Like rickettsia, *E. chaffeensis* is an obligate intracellular organism, primarily maintained in nature by the lone star tick (*Amblyomma americanum*) among white-tailed deer (Rikihisa, Y. 1999). *E. chaffeensis* is reported to be most prevalent in the southeastern U.S., notably in certain areas of Maryland (Stromdahl et al. 2000) and Missouri (Roland et al. 1998).

The lone star tick (*Amblyomma americanum*) occurs throughout Ohio as well as many other parts of the country and is considered the vector of *Ehrlichia chaffeensis*. Studies have shown a 15% infection rate of *E. chaffeensis* in *A. americanum* ticks from FL, AL, VA, AR, KS, NC, MD, and NJ (Stromdahl et al. 2001). However, to our knowledge, *E. chaffeensis* has yet to be reported in *A. americanum* ticks from Ohio. *A. americanum* ticks are frequently submitted to the Ohio Department of Health Vector-Borne Disease Program (ODH-VBDP) along with the American dog tick (*Dermacentor variabilis*). *D. variabilis* is the primary vector of *R. rickettsii*, the etiological agent of Rocky Mountain spotted fever. The ODH-VBDP was established in 1964 in response to tick-borne rickettsioses in Ohio and uses serological techniques to test *D. variabilis* ticks for SFG rickettsia. In the past, the *A. americanum* ticks submitted to the ODH-VBDP were considered non-vectors and were not routinely tested until recently.

In 1974 and 1985, previously unknown, non-pathogenic rickettsiae were isolated from *A. americanum* ticks in Tennessee and Missouri. Later analysis of the 17 kDa cell surface antigen gene and 16S rRNA gene in our laboratory showed that these two isolates were of identical genotypes and the name "R. amblyommii" was proposed (Stothard 1995). R. amblyommii sp. nov. has only been found in *A. americanum* ticks.

When constructing phylogenetic relationships, Stothard (Ph.D. dissertation, 1995) reported the 16S rRNA gene insufficient statistically for determining significant relationships beyond recognition between TG and SFG rickettsia. Stothard presented the use of the rickettsia-specific 17k Da cell surface antigen gene, which showed a much higher prevalence of nucleotide substitutions (12.5%

compared to 1.69% for the 16S rRNA gene). The 17 kDa antigen gene was also reported useful for identifying closely related species of rickettsia, especially those with overlapping geographic distributions and varying pathogenic potential (Stothard, 1995).

This study seeks to determine the prevalence of endosymbiont bacteria present in *A. americanum* ticks collected primarily from Ohio, as well as analyze the genetic diversity of putatively non-pathogenic rickettsiae in these ticks using the 17 kDa cell surface antigen gene.

MATERIALS AND METHODS

A total of 21 *A. americanum* ticks were provided to our laboratory from the ODH-VBDP. These ticks were submitted to the ODH from veterinarians, clinicians, and health departments primarily from Ohio in 2003. The ticks were stored at -20ºC until the DNA could be extracted. Ticks were sterilized in 70% ethanol and rinsed with distilled water. The ticks were then dissected using sterile, Admix "Nokor" 18 gauge needles to expose internal tissue to the extraction reagents. Genomic DNA was purified according to the manufacturers' protocol using either the PrepMan Ultra extraction solution (Applied Biosystems, Foster City, CA, USA) or a combination of the Isoquick Extraction Kit (Orca Research, Bothell, WA, USA) and the DNeasy Tissue Kit (Quiagen Sciences, Valencia, CA, USA). A 434bp fragment of the 17 kDa surface antigen protein was amplified using semi-nested polymerase chain reaction (Figure 2). Two µl of extracted DNA was amplified in 25 µl volumes consisting of 4.0 µl dNTPs, .25 µl of both 5' and 3' primers (17kDa-5'

GCTTTACAAAATTCTAAAAACCATATA and 17kDa-3' CTTGCCATTGTCCRTCAGGTTG), 1.25 µl $MgCl_2$, 2.5 µl reaction buffer, .25 µl *Taq* DNA polymerase, and 14.5 µl deionized distilled water. The semi-nested template DNA consisted of 2 µl of a 1:50 dilution of the first PCR. The remaining reaction components stayed the same with the exception of the nested 3' primer (17kDa-3'nest TCACGGCAATATTGACC). A negative control using deionized, distilled water was also included, and its primary PCR reaction was used in the semi-nested PCR to ensure absence of contaminants. The reaction parameters for both the primary and nested PCR consisted of a 10min denaturation step at 95ºC, followed by 35 cycles of 1min at 95ºC, 1min at 52ºC, and 1min at 72ºC. A final 15min extension step at 72ºC was included to make certain complete elongation of the fragments. All reactions took place in a Whatman-Biometra thermocycler (Biometra Biomedicine Analytik, Goettigen, Germany).

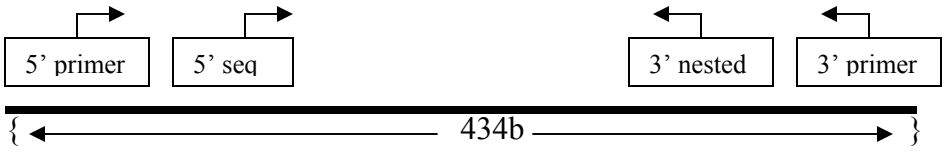

Figure 2: A simple schematic of the primers used in the primary, secondary, and sequencing PCR reactions of the 17 kDa antigen gene.

The fragment from the semi-nested PCR was purified using a Quiaquick PCR Purification Kit (Quiagen Sciences, Valencia, CA, USA) and then directly sequenced with the specific sequencing primer 17kDa 5'seq-GGTTCTCAATTYGG and Big Dye Terminator chemistry. All sequencing PCR reactions consisted of a 15sec denaturation at 94°C, 10sec annealing at 50°C, and 4min extension at 60°C. The DNA was then precipitated in 70% ethanol and analyzed on an ABI 310

automated DNA sequencer (Applied BioSystems). The sequences were then edited by eye in CLUSTAL and identification of the bacterial organism was performed using a nucleotide-nucleotide BLAST search in Genbank (http://www.ncbi.nlm.nih.gov).

RESULTS

A subset of 21 ticks submitted to the tick-screening program of the ODH-VBDP was examined for the presence of rickettsiae through a PCR detection assay followed by DNA sequence confirmation. Nine of the 21 ticks sampled (43%) contained amplicons that corresponded with the 434bp 17 kDa fragment that we targeted (table 1). A nucleotide-nucleotide BLAST search in Genbank using each of the 9 sequences of the PCR-positive samples returned 17 kDa rickettsiae species matches. Eight of the 9 samples (89%) were confirmed to be R. amblyommii and a single tick was found to carry the ancestral strain designated *R. bellii*.

Geographic data was only available on 10 of the 21 ticks surveyed (table 1). Nine of the 10 ticks were localized to the southern and eastern portions of Ohio (figure 3), with a single specimen originating from northern Ohio (Cuyahoga Co.). Of these 10 ticks with geographic data, three (30%) tested positive for rickettsiae. These three ticks were generally located in the southern half of the state, from Pike, Franklin, and Jackson counties respectively. This is the same region that is known to have a relatively high incidence of Rocky Mountain spotted fever and a high occurrence of rickettsia-positive *Dermacenter variabilis* ticks.

2003 ODH ID #	County	State	Date Collected	Sex	Percent Engorged	Notes	Extraction Method	Sequence
15	**********	AR	19-Mar-03	F	Trace	Attached to human	Q	belli
37	**********	NC	9-Mar-03	F	Trace	Attached to human	Q	ambly.
56	**********	KY	13-Apr-03	?	0	Attached to human	Q	ambly.
86	?	OH	23-Apr-03	F	0	Attached to human	Q	Ambly
344	Pike	OH	9-May-03	M	0	Attached to human	Q	ambly.
353	Hamilton	OH	6-May-03	Nymph	100	Dog (Attached)	Q	
362	Cuyahoga	OH	6-May-03	M	0	Not Attached	Q	
393	Jackson	OH	10-May-03	F	0	Attached to human	Q	
400	Lawrence	OH	28-Apr-03	M	0	Attached to human	Q	
407	Pike	OH	13-May-03	Nymph	0	Attached to human	Q	
853	Pike	OH	30-Jun-03	Nymph	0	Attached to human	AB	
854	Jackson	OH	30-Jun-03	Nymph	0	Attached to human	AB	ambly
916	Clark	OH	7-Jul-03	Nymph	Trace	Attached to human	AB	
917	Franklin	OH	15-Jul-03	M	0	**********	AB	ambly.
960	?	?	7-Jul-03	Nymph	0	Attached to human	AB	
961	?	?	7-Jul-03	Nymph	0	Attached to human	AB	
962	?	?	?	F	0	**********	AB	
810	**********	VA	June 22, 2003	Nymph	Trace	Attached to human		
924	**********	TN	July 14, 2003	Nymph	100	Attached to human		ambly
929	?	OH	?	Nymph	50	**********		
1064	**********	VA	July 29, 2003	Nymph	0	Attached to human		ambly

Table 1: This table describes demographics and data available for the selected *A. americanum* ticks. The data was provided by the Ohio Department of Health – Vector-Borne Disease Program. The ticks submitted from outside Ohio have the originating county denoted by '**********.' The abbreviation "Q" stands for the Quiagen DNeasy method of DNA extraction, and the abbreviation "AB" represents the PrepMan Ultra DNA extraction method.

Figure 3: Geographic distribution in Ohio of selected ticks tested for SFG rickettsia in 2003. The red circles indicate those ticks testing positive for "R. amblyommii." Those circles in white were determined negative for "R. amblyommii." Note: not all the ticks tested had geographic data available from the Department of Health.

DISCUSSION

These results indicate a high prevalence of rickettsiae-positive *A. americanum* ticks (43%) during the 2003 ODH-VBDP tick-screening program. This is significantly higher than the frequencies of rickettsiae (~ 4-5%) found in *Dermacentor* ticks. Rickettsia-positive *Amblyomma* ticks were identified to be associated with those same regions as rickettsia-positive *Dermacentor* ticks (Figure 4). However, the distribution and diversity of the rickettsial endosymbionts reported in each tick species is quite different (Tables 2a and 2b). *Amblyomma* ticks

were found to primarily host "R. amblyommii," with the exception of a single tick carrying *R. bellii* (Table 2a). Previous screenings of *Dermacentor* ticks, in contrast, have a more diverse array of rickettsiae, including *R. rickettsii*, *R. montanensis*, and *R. bellii*. Of these *Dermacentor* ticks, 59 (82%) were found to be carrying the strain *R. bellii*, which is a much higher frequency than that in our *Amblyomma* ticks (Table 2b). It appears that transfer of rickettsia strains between the two tick species is a rare occurrence, even though both *Amblyomma* and *Dermacentor* ticks are likely to share the same mammalian hosts. Additonal studies currently underway in our laboratory are being used to amplify both conserved and variable portions of the bacterial ftsZ cellular division gene. This gene has been shown to be a useful PCR detection assay of endosymbiotic bacteria that provides different sized amplicons visible between *Rickettsia rickettsii*, *Ehrlichia chaffeensis*, and *Anaplasma phagocytophilum* (Lee et al. 2003). Sequencing of these variable regions in the ftsZ gene will aid in developing a more specific PCR detection assay, as well as be a molecular marker for phylogenetic analysis and population structure of bacteria hosted by *Amblyomma* ticks.

Table 2a **Table 2b**

Nucleic acid identification of rickettsia strains from *A.americanum* tick screens, 2003		**Nucleic acid identification of rickettsia strains from *D. variabilis* from previous tick screens**		
R. amblyommii	*R. bellii*	*R. rickettsii*	*R. montanensis*	*R. bellii*
8	1	4	9	59

Tables 2a/2b: This table displays a simplified version of the frequency and distribution of infected *A. americanum* ticks compared to previous tick screens in our lab using *D. variabilis* ticks.

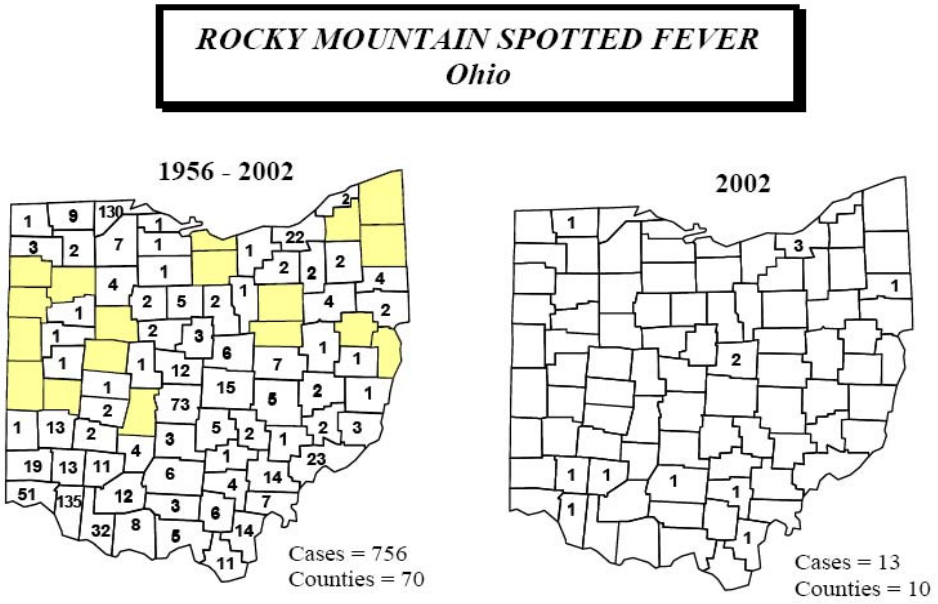

Figure 4: Geographic distribution of Spotted Fever in Ohio from 1956-2002. The black box indicates the region associated with rickettsia-positive ticks of both *Amblyomma* and *Dermacentor*. Information and Graphics are courtesy of the Ohio Department of Health.

Although studies have indicated that several species of ticks and mammals are infected with *E. chaffeensis*, *E. chaffeensis* is maintained primarily in nature by *A. americanum* among white-tailed deer (Rikihisa, Y. 1999). *E. chaffeensis* is the agent responsible for human monocytic ehrlichiosis and has not been reported in Ohio *A. americanum* ticks to date. The current study did not test for the presence of ehrlichial pathogens in *Amblyomma* ticks. However, Stromdahl et al. (2001) showed that a relative high frequency (15%) of *E. chaffeensis* infected *A. americanum* ticks in the eastern U.S., and that 2% of these ticks were co-infected with a second organism, *Borrelia burgdorferi*. It is therefore quite possible that similar circumstances can occur in Ohio *Amblyomma* ticks. This occurrence or co-occurrence of rickettsial and ehrlichial strains is the current subject of investigation in our laboratory.

In conclusion, our study uncovered a high frequency of rickettsiae in *A. americanum* ticks in Ohio. These ticks were not screened for rickettsial pathogens

prior to 2003. However, due to recent evidence of tick-borne rickettsioses and ehrlichioses, all ticks submitted to the ODH-VBDP are individually tested.

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